U.S. Application No.	International Application No. PCT/EP99/07049	Attorney Docket No. WWELL63.001APC

Date: March 22, 2002

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 USC 371

International Application No.:	PCT/EP99/07049
International Filing Date:	September 22, 1999
Priority Date Claimed:	None
Title of Invention:	RIBOZYMES FOR PREVENTION OF RESTENOSIS
Applicant(s) for DO/EO/US:	Gabriele Grassi, Anne Kuhn, and Reinhard Kandolf

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. (X) This is a **FIRST** submission of items concerning a filing under 35 USC 371.

2. () This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 USC 371.

3. (X) This express request to begin national examination procedures (35 USC 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 USC 371(b) and PCT Articles 22 and 39(1).

4. (X) A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. (X) A copy of the International Application as filed (35 USC 371(c)(2))

   a) () is transmitted herewith (required only if not transmitted by the International Bureau).
   b) (X) has been transmitted by the International Bureau.
   c) () a copy of Form PCT/1B/308 is enclosed.
   d) () is not required, as the application was filed in the United States Receiving Office (RO/US).

6. (X) A translation of the International Application into English (35 USC 371(c)(2)).

7. (X) Amendments to the claims of the International Application under PCT Article 19 (35 USC 371(c)(3))

   a) () are transmitted herewith (required only if not transmitted by the International Bureau).
   b) () have been transmitted by the International Bureau.
   c) () have not been made; however, the time limit for making such amendments has NOT expired.
   d) (X) have not been made and will not be made.

8. () A translation of the amendments to the claims under PCT Article 19 (35 USC 371(c)(3)).

9. () An oath or declaration of the inventor(s) (35 USC 371(c)(4)).

10. (X) A copy of the International Preliminary Examination Report with any annexes thereto, such as any amendments made under PCT Article 34.

11. () A translation of the annexes, such as any amendments made under PCT Article 34, to the International Preliminary Examination Report under PCT Article 36 (35 USC 371(c)(5)).

12. (X) An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

13. () An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

14. (X) A FIRST preliminary amendment.
() A SECOND or SUBSEQUENT preliminary amendment.

15. () A substitute specification.

16. () A power of attorney and/or address letter.

17. (X) International Application as published.

18. () The present application qualifies for small entity status under 37 C.F.R. § 1.27.

19. (X) Other Items or information:
1. Substitute Sequence Listing in computer readable form.
2. Paper copy of Substitute Sequence Listing.
3. Two (2) sheets of drawings.
4. International Search Report.

20. (X) A return prepaid postcard.

21. (X) The following fees are submitted:

				FEES
	BASIC FEE			$890
CLAIMS	**NUMBER FILED**	**NUMBER EXTRA**	**RATE**	
Total Claims	12 - 20 =	0 ×	$18	$0
Independent Claims	1 - 3 =	0 ×	$84	$0
Multiple dependent claims(s) (if applicable)			$280	$0
	TOTAL OF ABOVE CALCULATIONS		$890	
Reduction by 1/2 for filing by small entity (if applicable). Verified Small Entity statement must also be filed. (NOTE 37 CFR 1.9, 1.27, 1.28)			$0	
	TOTAL FEES ENCLOSED			$890

22. (X) The fee for later submission of the signed oath or declaration set forth in 37 CFR 1.492(e) will be paid upon submission of the declaration.

23. (X) A check in the amount of $890 to cover the above fees is enclosed.

24. () Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40 per property.

25. (X) The Commissioner is hereby authorized to charge only those additional fees which may be required, now or in the future, to avoid abandonment of the application, or credit any overpayment to Deposit Account No. 11-1410.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Mark R. Benedict
Reg. No. 44,531
Customer No. 20,995

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WWELL63.001APC PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant :	Grassi, et al.	) Group Art Unit: Unknown
Int'l Appl. No. :	PCT/EP99/07049	)
Int'l Filing Date:	September 22, 1999	)
For :	RIBOZYMES FOR PREVENTION OF RESTENOSIS	)
Examiner :	Unknown	)

PRELIMINARY AMENDMENT

United States Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

Preliminary to examination on the merits, please amend the above-captioned U.S. national phase application as follows:

IN THE SPECIFICATION:

On page 1 of the English language translation of the Specification, immediately after the Title of the Invention, please insert:

This is the U.S. national phase under 35 U.S.C. §371 of International Application PCT/EP99/07049, filed September 22, 1999.

On page 1 of the English language translation of the Specification immediately before the first paragraph which begins, "The present invention...", please insert:

Field of the Invention

On page 1 immediately after the first paragraph, which ends: "...angioplastic techniques.", please insert:

Background of the Invention

Int'l Appl. No. : PCT/EP99/07049
Int'l Filing Date: September 22, 1999

On page 3 immediately before the first full paragraph which begins, "In this connection,", please insert:

Summary of the Invention

On page 3 immediately after the first full paragraph, which ends "...immediately after balloon dilation.", please insert:

Brief Description of the Drawings

Fig. 1 shows the schematic structure of hammerhead ribozymes.

Fig. 2 shows a table containing the measured kinetic constants of the tested hammerhead ribozymes.

On page 3 immediately before the second full paragraph, which begins, "This therapeutic approach is based...", please insert:

Detailed Description of the Preferred Embodiment

On page 9, please amend the second full paragraph, which begins, "The examples below...", as follows: The examples below are illustrated on the basis of the attached drawings.

On page 8 immediately before Example 1, please delete the paragraph:

Fig. 1 shows the schematic structure of hammerhead ribozymes; and

Fig. 2 shows a table containing the measured kinetic constants of the tested hammerhead ribozymes.

On page 12, line 21, please delete the phrase: "SEQ ID No. 8 and 9,".

Please, remove pages 14 through 26.

On page 27, please delete the word "Claims" and substitute therefor:

WHAT IS CLAIMED IS:

IN THE CLAIMS:

Please, cancel the Claims 4 – 11, 15 - 22, 25, 27, and 28 without prejudice.

Please, amend the Claims as follows:

1. **(Amended)** A composition for preventing or ameliorating restenosis, comprising a catalytically acting RNA molecule which is directed against mRNA molecules coding for the cell cycle-relevant proteins cyclin E or E2F1.

2. **(Amended)** The RNA molecule of Claim 1, comprising a first sequence section binding to the mRNA molecules, a second sequence section binding to the mRNA molecules and a third sequence section which is located between the first and the second sequence section and catalyses site-specific cleavage of the bound mRNA molecules.

3. **(Amended)** The RNA molecule of Claim 2, wherein the first sequence section comprises a first nucleotide sequence of a nucleotide sequence pair or a nucleotide sequence that binds to sequences binding to the first nucleotide sequence, and the second sequence section comprises a second nucleotide sequence of said nucleotide sequence pair or a nucleotide sequence which binds to sequences binding to the second nucleotide sequence, said nucleotide sequence pair being selected from the group consisting of: SEQ ID No. 1 and SEQ ID No. 2, SEQ ID No. 3 and SEQ ID No. 4, SEQ ID No. 5 and SEQ ID No. 6, SEQ ID No. 7 and SEQ ID No. 8, SEQ ID No. 9 and SEQ ID No. 10, SEQ ID No. 11 and SEQ ID No. 12, SEQ ID No. 13 and SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16, and SEQ ID No. 17 and SEQ ID No. 18.

12. **(Amended)** The RNA molecule of Claim 3, wherein the third sequence section comprises at its 3' end the sequence of SEQ ID No. 19, and at its 5' end the sequence of SEQ ID No. 20.

13. **(Amended)** The RNA molecule of Claim 12, wherein the third sequence section comprises the sequence of SEQ ID No. 21.

14. **(Amended)** The RNA molecule of Claim 1, wherein it comprises a sequence selected from the group consisting of: SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, and SEQ ID No. 30.

23. **(Amended)** A DNA molecule comprising a sequence coding for the RNA molecule of Claim 1.

24. **(Amended)** A vector plasmid, comprising the DNA sequence of Claim 23, and a promoter inserted in front of the DNA sequence section.

26. **(Amended)** A kit for preparing a vector plasmid, comprising the DNA molecule of Claim 23.

29. **(New)** The RNA molecule of Claim 2, wherein the third sequence section comprises at its 3' end the sequence of SEQ ID No. 19, and at its 5' end the sequence of SEQ ID No. 20.

30. **(New)** The RNA molecule of Claim 29, wherein the third sequence section comprises the sequence of SEQ ID No. 21.

31. **(New)** The vector plasmid of Claim 24, wherein said promoter is expressed exclusively in smooth muscle cells.

IN THE SEQUENCE LISTING

Please, insert the Substitute Sequence Listing, that has been conformed to the U.S. practice.

REMARKS

Claims 4 – 11, 15 - 22, 25, 27, and 28 have been cancelled without prejudice. Claims 1, 2, 3, 12, 13, 14, 23, 24, and 26 have been amended to more precisely claim the invention according to conventional practice before the United States Patent and Trademark Office. New Claims 29 - 31 have been added. Support for new Claims 29 –31 can be found in original Claims and the Specification. As a result Claims 1, 2, 3, 12, 13, 14, 23, 24, 26, 29, 30 and 31 are presented for examination. Submitted herewith is the paper copy and the C.R.F. of Substitute Sequence Listing that has been conformed to the U.S. practice. Field <110> of the Sequence Listing now recites the inventors as Applicants: Gabrielle Grassi, Anne Kuhn, and Reinhard Kandolf. Fields <150> and <151> have been added to reflect the priority application number: PCT/EP99/07049, and priority application filing date: September 22, 1999, respectively.

The changes made to the Specification and the Claims by the current amendment, including insertions and **[deletions]**, are shown on attached sheets entitled **VERSION WITH MARKINGS TO SHOW CHANGES MADE**, which follow the signature page of this amendment.

No new matter is being added herewith.

Should there be any questions concerning this application, the Examiner is respectfully invited to contact the undersigned attorney at the telephone appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Int'l Appl. No. : PCT/EP99/07049
Int'l Filing Date: September 22, 1999

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: 3/22/02

By: _____

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification.

On page 12:

The experimentally confirmed binding arm pairs I and II are represented in the attached sequence listing as sequences SEQ ID No. 1 and 2, SEQ ID No. 3 and 4, SEQ ID No. 5 and 6, SEQ ID No. 7 and 8, **[SEQ ID No. 8 and 9,]** SEQ ID No. 9 and 10, SEQ ID No. 11 and 12, SEQ ID No. 13 and 14, SEQ ID No. 15 and 16, and SEQ ID No. 17 and 18, the sequence having the odd SEQ ID in each case denoting the binding arm at the 5' end and the sequence having the even SEQ ID denoting the binding arm at the 3' end of the ribozyme.

In the Claims:

1. **(Amended)** A composition for preventing or ameliorating restenosis, comprising a catalytically acting RNA molecule which is directed against mRNA molecules coding for the cell cycle-relevant proteins cyclin E or E2F1.

2. **(Amended)** The RNA molecule of claim 1, **[having]** comprising a first sequence section binding to the mRNA molecules, a second sequence section binding to the mRNA molecules and a third sequence section which is located between the first and the second sequence section and catalyses site-specific cleavage of **[a]** the bound mRNA **[molecule]** molecules.

3. **(Amended)** The RNA molecule of Claim 2**[characterized in that]**, wherein the first sequence section **[has the sequence SEQ ID No. 1 or a sequence which]** comprises a first nucleotide sequence of a nucleotide sequence pair or a nucleotide sequence that binds to sequences binding to the first nucleotide sequence **[SEQ ID No. 1]**, and the second sequence section **[has the sequence SEQ ID No. 2 or a]**comprises a second nucleotide sequence of said nucleotide sequence pair or a nucleotide sequence which binds to sequences binding to the **[sequence SEQ ID No. 2.]** second nucleotide sequence, said nucleotide sequence pair being selected from the group consisting of: SEQ ID No. 1 and SEQ ID No. 2, SEQ ID No. 3 and SEQ ID No. 4, SEQ ID No. 5 and SEQ ID No. 6, SEQ ID No. 7 and SEQ ID No. 8, SEQ ID No. 9 and SEQ ID No. 10, SEQ ID No. 11 and SEQ ID No. 12, SEQ ID No. 13 and SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16, and SEQ ID No. 17 and SEQ ID No. 18.

4. **(Cancelled)**

5. **(Cancelled)**

6. **(Cancelled)**

7. **(Cancelled)**

8. **(Cancelled)**

9. **(Cancelled)**

10. **(Cancelled)**

11. **(Cancelled)**

12. **(Amended)** The RNA molecule of **[any of claims 2 to 11, characterized in that]** Claim 3, wherein the third sequence section **[has]** comprises at its 3' end the sequence of SEQ ID No. 19, and at its 5' end the sequence of SEQ ID No. 20.

13. **(Amended)** The RNA molecule of **[claim 12, characterized in that]** Claim 12, wherein the third sequence section **[has]** comprises the sequence of SEQ ID No. 21.

14. **(Amended)** The RNA molecule of Claim 1,**[characterized in that it has the sequence SEQ ID No. 22.]** wherein it comprises a sequence selected from the group consisting of: SEQ ID No. 22, SEQ ID No. 23, SEQ ID No. 24, SEQ ID No. 25, SEQ ID No. 26, SEQ ID No. 27, SEQ ID No. 28, SEQ ID No. 29, and SEQ ID No. 30.

15. **(Cancelled)**

16. **(Cancelled)**

17. **(Cancelled)**

18. **(Cancelled)**

19. **(Cancelled)**

20. **(Cancelled)**

21. **(Cancelled)**

22. **(Cancelled)**

23. **(Amended)** A DNA molecule comprising a **[having at least one]** sequence **[section]** coding for the RNA molecule of **[any of claims 1 to 22]** Claim 1.

24. **(Amended)** A vector plasmid, comprising **[having at least one]** the DNA sequence of Claim 23, and a promoter inserted in front of the DNA sequence section.

25. **(Cancelled)**

26. **(Amended)** A kit **[Kit containing]** for preparing a vector plasmid, comprising the DNA molecule of Claim 23.

27. **(Cancelled)**

28. **(Cancelled)**

WWELL63.001APC PATENT

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Ribozymes for prevention of restenosis

The present invention relates to arteriosclerosis therapy and in particular to prevention of restenosis of blood vessels after stenoses that have been treated using angioplastic techniques.

Coronary heart disease and other arteriosclerotic disorders are the leading cause for morbidity and mortality in the industrialized Western world. On average, every other patient suffers from a cardiovascular disorder as a result of arteriosclerotic vascular changes.

Besides surgery, in most cases bypass surgery, angioplastic techniques in which the vascular constriction or occlusion, i.e. stenosis, is removed mechanically, for example, by means of balloon dilatation (PTCA), has gained strongly in importance in recent years. In connection with this treatment, in order to counteract a renewed vascular occlusion, frequently so-called stents are implanted into the vessels in order to keep their lumina open and thus to prevent restenosis.

Despite the latest catheterization techniques and stent implants and also pharmacological intervention, however, the above-mentioned therapeutic measures are substantially limited by restenosis whose incidence is 30-50%. An abnormal transient growth stimulation of vascular smooth muscle cells must be regarded as the main cause of this restenosis. The uncontrolled growth of the vascular smooth muscle cells which results therefrom and their migration into the intimal space and also a reduction in death processes then lead to stenosis, due to the resulting large number of intimal cells. This large number of intimal smooth muscle cells is the now accepted key finding in human restenotic tissues.

For the individual patient, restenosis requires a renewed traumatizing, possibly surgical intervention with the usual risks of complication. This alone necessitates novel more effective therapeutic approaches to prevent restenosis.

Furthermore, the dimension of a reduction in cost- and manpower-intensive acute and follow-up measures due to such novel therapeutic approaches is becoming apparent in view of a worldwide angioplasty frequency of approximately one million interventions per year. Based on the model character of restenosis for accelerated arteriosclerosis, it is also possible to use these novel therapeutic approaches for treating bypass/shunt arteriosclerosis, transplant arteriopathy and also clinically particularly early or progressive forms of arteriosclerosis by way of a primary prevention with comparable effectiveness. In addition to the intraluminal treatment of defined target stenoses, percutaneous non-surgical catheterization techniques also provide the option of selective administration

of protective substances into the infarcted vessel with the aim of reducing the infarct area or preventing the formation of aneurysms.

In this connection, the present invention proposes the prevention of restenosis by inhibiting proliferation of the vascular smooth muscle cells, using therapeutic genes which induce cell cycle arrest and may be administered immediately after balloon dilatation.

This therapeutic approach is based on the finding that the massive local increase in various growth factors after balloon dilatation, with synthesis of G1-phase cyclins resulting therefrom, causes reentry of the smooth muscle cells into the cell cycle and thus their uncontrolled proliferation.

At a conference from 23rd to 27th September 1998 in Cold Spring Harbor, New York, USA, the inventors of the present application already suggested in this connection the use of hammerhead ribozymes, i.e. catalytic RNA molecules, which are directed against mRNA molecules coding for the cell cycle-relevant proteins cyclin E or E2F1; Grassi et al., Pathol Res Pract 1998, 194/4: 267 (Abstract 214). This approach is based on the finding that it is possible to achieve efficient inhibition of proliferation if the production of cell cycle-relevant proteins which are involved in a great variety of regulatory pathways is prevented.

This applies to the proteins cyclin E and E2F1 which are part of many regulatory pathways, among other things of a regulatory feedback mechanism in which cyclin E via interaction

with other products releases the transcription factor E2F1 in active form. E2F1 in turn activates the transcription of genes whose products are essential for the S phase and stimulates both cyclin E transcription and transcription of its own gene. A combined inactivation of cyclin E and E2F1 is therefore a particularly efficient method of inducing cell cycle arrest.

The functions and sequences of cyclin E and E2F1 are described in various publications, for example by Koff et al., Cell (1991), volume 66, 1217-1228, Geng et al., Oncogene (1996), volume 12, 1173-1180, Helin et al., Cell (1992), volume 70, 337-350, Ohtani et al., PNAS (1995), volume 92, 12146-50. Inactivation of the said proteins can efficiently prevent progression of vascular smooth muscle cells through the cell cycle and transition from the G1 into the S phase of the cell cycle does not take place.

Various techniques are available for inactivating genes and/or products thereof, but the use of catalytic RNA molecules, so-called ribozymes, however has the advantage that on the one hand said ribozymes break down the mRNA molecules coding for the proteins cyclin E and E2F1 because of their catalytic activity in trans and on the other hand a selective effect is achieved because of their sequence-specific interaction.

The catalytic RNA molecules used within the scope of the invention are so-called hammerhead ribozymes, as described, for example, in the review by Symons: Small Catalytic RNAs, Annu Rev Biochem (1992), volume 61, 641-671. Due to their ability to bind complementary RNA molecules via base pairing and to de-

stroy them by site-specific cleavage, hammerhead ribozymes are suitable candidates for preparation of therapeutics.

In principle, hammerhead ribozymes are constructed such that they have two sequence sections, the so-called binding arms, which are responsible for specific binding to the target RNA, and a more or less conserved catalytically acting sequence section which is arranged between the two binding arms. This catalytic sequence section contains two conserved regions at the 3′ and 5′ ends, and another sequence section which is at least partly double-stranded and can be, at least theoretically, of any length and sequence is present between these two regions. Binding to the target RNA results in the formation of the typical hammerhead as shown in Fig. 1. An arrow indicates the cleavage site and boxes highlight the conserved regions.

As already mentioned, the specificity of the hammerhead ribozymes is determined by the sequences of the binding arms which in the region of the target triplet bind to the RNA molecule to be cut. The determination of binding arms for ribozymes directed against target RNA thus requires firstly identification of single-stranded regions in the target RNA molecule and also selection of suitable cleavage site triplets with subsequent determination of the binding arm sequences. In particular in the case of complicated RNA molecules with relatively long strands, such as, for example, the mRNA for cyclin E and E2F1, the binding arm sequences cannot simply be determined on the basis of the possibly known sequence of the RNA molecule, since the secondary structure thereof and also the single-stranded regions freely accessible to the ribozymes cannot readily be derived from the sequence. Although there are computer models

which may be used for determining the secondary structure of long RNA molecules, the inventors of the present application have found that this method cannot successfully be applied for determining suitable cleavage sites in mRNA molecules for cyclin E and E2F1.

The inventors of the present invention have therefore employed the RNase H technique described in more detail in the embodiments, in order to determine suitable binding sites for hammerhead ribozymes. After the possible target sites had been determined experimentally, possible binding arm sequences had to be derived therefrom and the corresponding ribozymes had to be prepared. In this connection, it emerged that the length of the binding arms is crucial for the efficacy of the catalytic activity of the ribozymes so that again only an experimental determination of efficient ribozymes was possible.

The sequences listed in the attached sequence listing, SEQ ID No. 1 and SEQ ID No. 2, SEQ ID No. 3 and SEQ ID No. 4, SEQ ID No. 5 and SEQ ID No. 6, SEQ ID No. 7 and SEQ ID No. 8, SEQ ID No. 9 and SEQ ID No. 10 and SEQ ID No. 11 and SEQ ID No. 12 represent binding arm pairs for enzymes against cyclin E mRNA, and the sequences SEQ ID No. 13 and SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16 and SEQ ID No. 17 and SEQ ID No. 18 represent binding arm pairs for ribozymes against E2F1 RNA. Ribozymes with these binding arms have a particularly good catalytic effect against the said mRNA molecules, as the inventors of this application were able to show experimentally.

Instead of the sequences indicated, the binding arms may also have sequences which bind to sequences binding to the sequences mentioned in each case.

In this connection it is preferred, if the catalytic third sequence section has at its 3′ end the sequence SEQ ID No. 19 and at its 5′ end the sequence SEQ ID No. 20, this third sequence section preferentially comprising the sequence SEQ ID No. 21.

The attached sequence listing contains the sequences of tested ribozymes against cyclin E as SEQ ID No. 22 to SEQ ID No. 27 and of those against E2F1 as SEQ ID No. 28 - SEQ ID No. 30. Table 1 in the detailed description indicates the kinetic constants for these ribozymes which are among the best constants described for hammerhead ribozymes.

Although it is in principle possible to introduce the catalytic RNA molecules into the target cells using an appropriate target cell-specific transport system, it is more advantageous to use DNA molecules for this, which comprise a sequence section coding for the above-mentioned catalytic RNA molecules. This DNA sequence section may be part of a vector plasmid or may be used for generating a vector, preferably an adenoviral vector for application in gene therapy.

Compared with other methods, introduction of the foreign DNA by means of recombinant adenoviruses in particular has the advantage that adenoviruses infect target cells with high efficiency and there is no risk of insertion mutagenesis.

The applicability in principle of somatic gene therapy for cardiovascular disorders by means of replication-incompetent adenoviral constructs was described, for example, by Barr et al., J. Cell Biochem (1993), volume 17D, 192.

In general, the therapeutic effect can thus be achieved either via selective transfer systems or via cell-specific gene expression, and a selective and efficient expression via tissue-specific expression of the therapeutic genes is achieved by a promoter which is expressed exclusively in smooth muscle cells and has a high expression rate.

In this connection the present invention also relates to a therapeutic composition comprising the novel catalytic RNA molecule or the novel DNA molecule or the novel vector plasmid, respectively.

In order to prepare specific vectors or vector plasmids, the present invention further relates to a kit containing the novel DNA molecule which has at least one sequence section coding for the novel RNA molecule.

In general, the present invention relates to the use of the novel catalytically acting RNA molecule, the novel DNA molecule and/or the novel vector plasmid for prevention of restenosis by means of inhibiting proliferation of vascular smooth muscle cells.

It is understood that the features mentioned above and still to be illustrated below can be used not only in the combination indicated in each case but also in other combinations

or on their own, without going beyond the scope of the present invention.

Further features and advantages of the present invention arise from the following description of preferred embodiments.

The examples below are illustrated on the basis of the attached drawing in which:

Fig. 1 shows the schematic structure of hammerhead ribozymes; and

Fig. 2 shows a table containing the measured kinetic constants of the tested hammerhead ribozymes.

Example 1:

Structure of hammerhead ribozymes

Fig. 1 shows the basic structure of a hammerhead ribozyme, as can be found, for example, in the publication by Symons mentioned at the outset.

Fig. 1 shows a ribozyme hybridized with a substrate (target mRNA), whereby two double-stranded regions are formed between the substrate and the ribozyme, between which regions the cleavage site indicated by an arrow can be found on the substrate side. The ribozyme side comprises the catalytic region III located between the two sequence sections I and II which are denoted "binding arm", which region III comprises

conserved regions and variable regions, the conserved regions being highlighted by boxes.

On the substrate side, a box likewise highlights the target triplet NUH. It should be noted that N denotes any of the four nucleotides, H denotes any of the nucleotides A, U and C, Y denotes a pyrimidine and R a purine.

The catalytic sequence section of the ribozyme comprises at its 3' end the sequence SEQ ID No. 19 and at its 5' end the sequence SEQ ID No. 20 of the attached sequence listing. SEQ ID No. 21 of the attached sequence listing shows an example of the catalytic sequence section III and also indicates the double-stranded region and the loop region from the third sequence section III by way of example with defined nucleotides.

The specificity of the ribozyme of Fig. 1 for the target mRNA results from the specific sequence of binding arms I and II which must be selected such that they flank a target triplet NUH on the substrate, as indicated.

Example 2 describes how to determine the binding arm sequences.

Example 2

RNase H mapping of the ribozyme-accessible cleavage sites of cyclin E and E2F1

The cleavage sites of cyclin E and E2F1, which are accessible for hammerhead ribozymes, cannot be determined immedi-

ately on the basis of the known mRNA sequences for cyclin E (Koff et al., loc. cit., Geng et al., loc. cit.) and E2F1 (Helin et al., loc. cit.) or on the basis of mathematical folding models of these mRNAs so that experimental methods had to be applied.

The accessible cleavage sites were therefore determined by applying the method of RNase H mapping, as described by Zarinkar et al., Nature Struct Biol (1996) volume 3, 432 and Lapham et al., RNA (1996), volume 2, 289. To this end, completely randomized nonameric oligodeoxynucleotides were incubated with the 5′ end-radiolabelled target RNA, with complementary base pairing taking place only in open RNA structures, i.e. single-stranded "loops", but not in closed regions, the so-called stems. Based on the ability of RNase H to cleave such RNA/DNA hybrids and depending on the RNase H cleavage sites, a mixture of RNA fragments is obtained whose lengths reflect those positions of the open regions, which are in principle accessible for ribozymes.

The length of the RNase H fragments was then determined electrophoretically, and it was then possible via the use of sequence-specific oligodeoxynucleotides to confirm possible ribozyme cleavage sites.

For cyclin E, RNase H mapping was carried out using class III and class I transcripts which differ from one another only in the absence of two exons in the class I transcript (exons I and II).

For E2F1 transcripts, too, two RNAs of different lengths were used, but the truncated transcript has no physiological importance.

The open RNA regions determined experimentally for cyclin E and E2F1 were compared with various computer-assisted folding analyses, and it was found that it was not possible to find an optimal agreement between experimentally mapped cleavage sites and the predicted structure.

In knowledge of the target triplet NUH mentioned in Example 1, various binding arm I and II sequences were determined for the open RNA regions, which were then used to prepare and test hammerhead ribozymes, as is described in Example 3 below.

It was found in these experiments that the length of the binding arms is one of the deciding factors for the efficiency of the catalytic effect of the ribozymes synthesized in this way, and it was furthermore found that the ribozymes generated were not able to recognize and cleave any possible cleavage site.

The experimentally confirmed binding arm pairs I and II are represented in the attached sequence listing as sequences SEQ ID No. 1 and 2, SEQ ID No. 3 and 4, SEQ ID No. 5 and 6, SEQ ID No. 7 and 8, SEQ ID No. 8 and 9, SEQ ID No. 9 and 10, SEQ ID No. 11 and 12, SEQ ID No. 13 and 14, SEQ ID No. 15 and 16 and SEQ ID No. 17 and 18, the sequence having the odd SEQ ID in each case denoting the binding arm at the 5′ end and the sequence having the even SEQ ID denoting the binding arm at the 3′ end of the ribozyme.

Example 3

Determination of the activity of the hammerhead ribozymes

On the basis of binding arms SEQ ID No. 1 to 18 and the catalytically acting sequence SEQ ID No. 21, nine hammerhead ribozymes were prepared whose sequences are listed in the attached sequence listing under SEQ ID No. 22 to SEQ ID No. 30.

The activity was determined by incubating transcribed RNAs for E2F1, class I cyclin E and class III cyclin E with the relevant ribozyme in the presence of ^{32}P-UTP, and determining the kinetic constant Kcat/Km (in 10^4 M^{-1} min^{-1}). The in vitro activity of the hammerhead ribozymes having the sequences SEQ ID No. 22-30 is shown in Table 1 which is contained in Fig. 2.

Example 4

Construction of replication-incompetent adenoviruses

DNA molecules coding for the catalytic RNA molecules of Examples 2 and 3 are cloned into an adenoviral vector, the recombinant adenoviruses having been made replication incompetent by mutation or deletion.

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Claims

1. Catalytically acting RNA molecule which is directed against mRNA molecules coding for the cell cycle-relevant proteins cyclin E or E2F1.

2. The RNA molecule of claim 1, having a first sequence section binding to the mRNA molecules, a second sequence section binding to the mRNA molecules and a third sequence section which is located between the first and the second sequence section and catalyses site-specific cleavage of a bound mRNA molecule.

3. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 1 or a sequence which binds to sequences binding to the sequence SEQ ID No. 1, and the second sequence section has the sequence SEQ ID No. 2 or a sequence which binds to sequences binding to the sequence SEQ ID No. 2.

4. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 3 or a sequence which binds to sequences binding to the sequence SEQ ID No. 3, and the second sequence section has the sequence SEQ ID No. 4 or a sequence which binds to sequences binding to the sequence SEQ ID No. 4.

5. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 5 or a

sequence which binds to sequences binding to the sequence SEQ ID No. 5, and the second sequence section has the sequence SEQ ID No. 6 or a sequence which binds to sequences binding to the sequence SEQ ID No. 6.

6. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 7 or a sequence which binds to sequences binding to the sequence SEQ ID No. 7, and the second sequence section has the sequence SEQ ID No. 8 or a sequence which binds to sequences binding to the sequence SEQ ID No. 8.

7. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 9 or a sequence which binds to sequences binding to the sequence SEQ ID No. 9, and the second sequence section has the sequence SEQ ID No. 10 or a sequence which binds to sequences binding to the sequence SEQ ID No. 10.

8. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 11 or a sequence which binds to sequences binding to the sequence SEQ ID No. 11, and the second sequence section has the sequence SEQ ID No. 12 or a sequence which binds to sequences binding to the sequence SEQ ID No. 12.

9. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 13 or a sequence which binds to sequences binding to the sequence SEQ ID No. 13, and the second sequence section has the sequence SEQ

ID No. 14 or a sequence which binds to sequences binding to the sequence SEQ ID No. 14.

10. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 15 or a sequence which binds to sequences binding to the sequence SEQ ID No. 15, and the second sequence section has the sequence SEQ ID No. 16 or a sequence which binds to sequences binding to the sequence SEQ ID No. 16.

11. The RNA molecule of claim 2, characterized in that the first sequence section has the sequence SEQ ID No. 17 or a sequence which binds to sequences binding to the sequence SEQ ID No. 17, and the second sequence section has the sequence SEQ ID No. 18 or a sequence which binds to sequences binding to the sequence SEQ ID No. 18.

12. The RNA molecule of any of claims 2 to 11, characterized in that the third sequence section has at its 3′ end the sequence SEQ ID No. 19, and at its 5′ end the sequence SEQ ID No. 20.

13. The RNA molecule of claim 12, characterized in that the third sequence section has the sequence SEQ ID No. 21.

14. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 22.

15. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 23.

16. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 24.

17. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 25.

18. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 26.

19. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 27.

20. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 28.

21. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 29.

22. The RNA molecule of claim 1, characterized in that it has the sequence SEQ ID No. 30.

23. A DNA molecule having at least one sequence section coding for the RNA molecule of any of claims 1 to 22.

24. A vector plasmid, having at least one DNA sequence section coding for the RNA molecule of any of claims 1 to 22, and a promoter inserted in front of the DNA sequence section.

25. A therapeutic composition having an RNA molecule of any of claims 1 to 22, or a DNA molecule of claim 23, or a vector plasmid of claim 24.

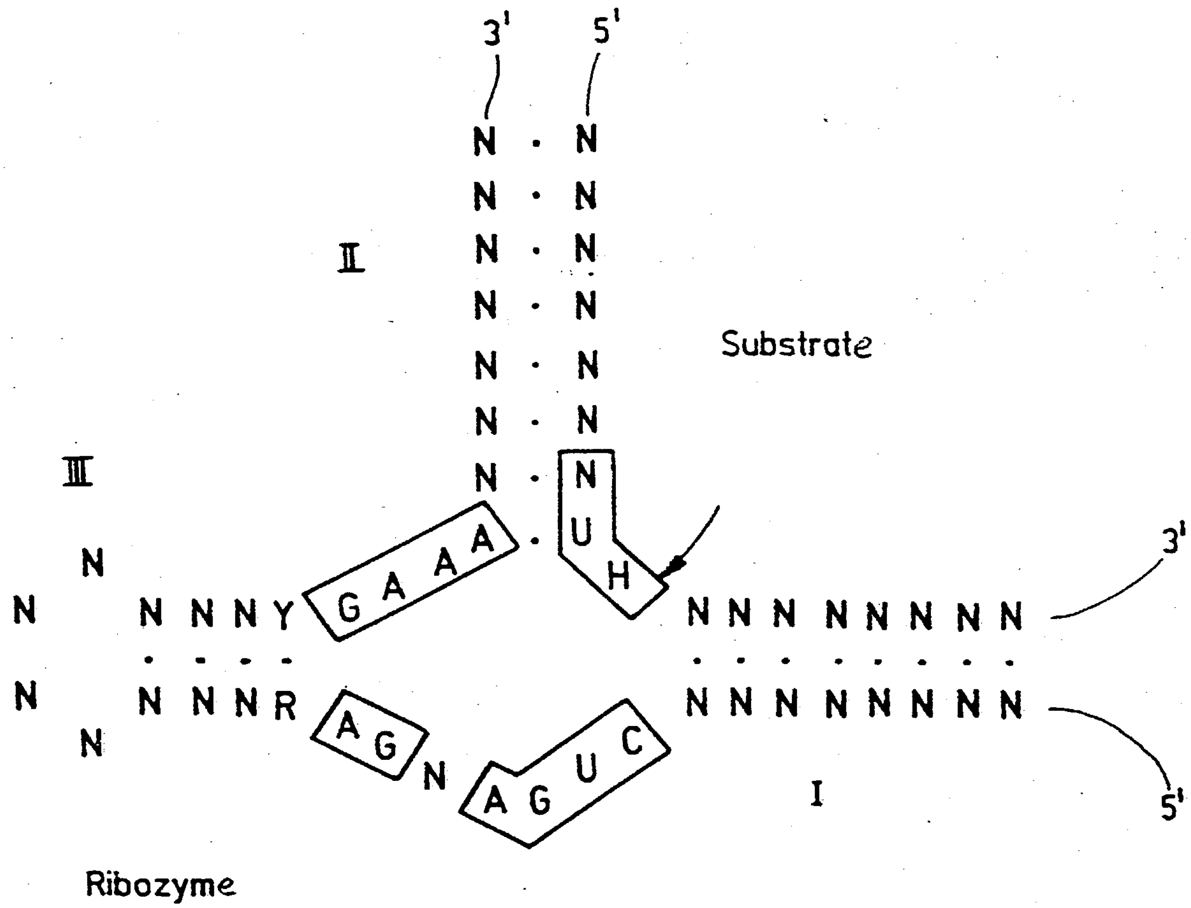
26. Kit containing the DNA molecule of claim 23.

27. A use of the RNA molecule of any of claims 1 to 22 and/or of the DNA molecule of claim 23 for generating a vector for gene therapy.

28. A use of the RNA molecule of any of claims 1 to 22 and/or of the DNA molecule of claim 23 and/or of the vector plasmid of claim 24 for inhibiting restenosis of angioplastically treated blood vessels.

Abstract

A catalytically acting RNA molecule is proposed which is directed against mRNA molecules coding for the cell cycle-relevant proteins cyclin E and E2F1. The catalytically acting RNA molecule is a hammerhead ribozyme which induces cell cycle arrest of vascular smooth muscle cells to prevent restenosis.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5'
I
Ribozyme

Fig.1

Table 1

Ribozyme	Target	cleavage site	K_m	K_{cat}	K_{cat}/K_m
	Cyclin E		*nM*	*10^{-3} min^{-1}*	*$10^4 M^{-1}$ min^{-1}*
SEQ ID No. 22	class III	439	13.9±1.88	9.7±0.3	73±7.76
	class I	230	13.07±0.95	8.22±0.46	63.7±3.7
SEQ ID No. 23	class III	439	6.18±0.43	4.7±0.44	75.9±1.8
	class I	230	7.98±0.53	5.25±0.35	65.9±0.2
SEQ ID No. 24	class III	1277	14.86±2.01	6.64±0.42	46.5±5.48
	class I	1068	18.15±1.88	6.91±0.36	38.87±3.26
SEQ ID No. 25	class III	1277	11.04±1.66	5.93±0.29	53.9±2.58
	class I	1068	11.63±1.89	4.59±0.9	39.3±1.4
SEQ ID No. 26	class III	221	51.46±0.24	7.03±0.89	13.3±1.85
SEQ ID No. 27	class III	422	41.3±6.02	10.93±0.64	27±2.88
	class I	213	34.09±3.56	10.1±1.27	29.6±2.9
	E2F1				
SEQ ID No. 28	full length	732	20.38±1.43	11.1±0.45	56.18±2.75
	shortened	242	28.34±1.94	14.76±1.8	52.17±5.8
SEQ ID No. 29	full length	732	24.69±0.01	12.15±0.55	49.35±2.25
	shortened	242	22.24±0.23	14.99±0.41	66.5±1.15
SEQ ID No. 30	full length	1180	23.53±0.16	8.4±0.3	36±1.1
	shortened	690	38.9±5.34	14.45±2.7	36.85±1.9

Fig. 2

DECLARATION AND POWER OF ATTORNEY - USA PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled RIBOZYMES FOR PREVENTION OF RESTENOSIS the specification of which was described and claimed in PCT International Application No. EP99/07049 filed on September 22, 1999.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above;

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, § 1.56;

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent, design or inventor's certificate or any PCT international application(s) listed below and have also identified below any foreign application(s) for patent, design or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed for the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 37 U.S.C. § 119
			o YES NO o

POWER OF ATTORNEY: I hereby appoint the registrants of Knobbe, Martens, Olson & Bear, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, California 92660, Telephone (949) 760-0404, Customer No. 20,995.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

Full name of first inventor: **Gabriele GRASSI**

Inventor's signature Gabriele Grassi Day 17 Month June Year 2002

Residence (city and country): Tübingen, Germany DEX

Citizenship: German

Post Office Address: Mohlstrasse 52, D-72074 Tübingen, Germany

2-00

Full name of second inventor: **Anne KUHN**

Inventor's signature Anne Kuhn Day 17 Month June Year 2002

Residence (city and country~~)~~: Tübingen, Germany DEX

Citizenship: German

Post Office Address: Marienstraße 33, D-72072 Tübingen, Germany

3-00

Full name of third inventor: **Reinhard KANDOLF**

Inventor's signature Kandolf Day 17 Month June Year 2002

Residence (city and country): Hechingen, Germany DEX

Citizenship: German

Post Office Address: Untere Dornäcker 49, D-72379 Hechingen, Germany

Send Correspondence To:
KNOBBE, MARTENS, OLSON & BEAR, LLP
Customer No. 20,995

O:\DOCS\MXG\MXG-1276.DOC:vb
040402

ESTABLISHMENT OF RIGHT OF ASSIGNEE TO TAKE ACTION
AND
REVOCATION AND POWER OF ATTORNEY

To the Commissioner of Patents and Trademarks:

The undersigned is empowered to act on behalf of the assignee indicated below (the "Assignee"). The original assignment of the U.S. Application No. 10/089,127, filed March 22, 2002, for Letters Patent for the invention in RIBOZYMES FOR PREVENTION OF RESTENOSIS from the inventors to the Assignee is being submitted herewith for recordation by the Assignment Branch. A true copy of this Assignment is attached hereto. This Assignment represents the entire chain of title of this invention from the Inventor(s) to the Assignee. I have reviewed this Assignment, and to the best of the Assignee's knowledge and belief, the Assignee is the owner of the entire right, title, and interest in the above-referenced application.

I declare that all statements made herein of my own knowledge are true, and that all statements made upon information and belief are believed to be true, and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that willful, false statements may jeopardize the validity of the application, or any patent issuing thereon.

The undersigned hereby revokes any previous powers of attorney in the subject application, and hereby appoints the registrants of Knobbe, Martens, Olson & Bear, LLP, 620 Newport Center Drive, Sixteenth Floor, Newport Beach, California 92660, Telephone (949) 760-0404, **Customer No. 20,995**, as its attorneys with full power of substitution and revocation to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected herewith. This appointment is to be to the exclusion of the inventor(s) and his attorney(s) in accordance with the provisions of 37 C.F.R. § 3.71.

Please use **Customer No. 20,995** for all communications.

Assignee: Eberhard-Karls-Universität Tübingen
Universitätsklinikum

By: 

Printed Name: Hans-Rüdiger Strehl

Title: Administrative Director

Address: Geissweg 3, D-72076 Tübingen
GERMANY

Dated: June, 21, 2002

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